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ORIGINAL ARTICLE

The effects of immunosuppressants on vascular function, systemic oxidative stress and inflammation in rats

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Summary

Immunosuppressants have been associated with increased cardiovascular disease risk. We determined the effects of calcineurin and mammalian target of rapamycin (mTOR) inhibitor administration on endothelial dysfunction and associated inflammation and oxidative stress in adult rats. Cyclosporine A (low and high dose), sirolimus, tacrolimus, everolimus and placebo were administered to 8-week-old male Wistar rats for 10 consecutive days. Aortic vascular endothelial and smooth muscle function were assessed *ex vivo* in organ baths. Maximal aortic contraction to noradrenaline in sirolimus-treated rats was significantly greater than cyclosporine groups, everolimus and placebo, whereas endothelial-dependent relaxation was significantly impaired with cyclosporine and tacrolimus compared with everolimus. Endothelial-independent relaxation was impaired in tacrolimus-treated rats compared with low dose cyclosporine, everolimus and sirolimus. Sirolimus was associated with a reduction in plasma interleukin (IL)-1β and tumour necrosis factor (TNF)-α and higher levels of catalase and total antioxidant status. In nontransplanted rats, vascular dysfunction was evident following administration of cyclosporine A, sirolimus and tacrolimus, whereas everolimus did not compromise aortic endothelial or smooth muscle function. At the doses administered in this model, the immunosuppressants exerted varying effects on vascular function.

Introduction

Calcineurin and mammalian target of rapamycin (mTOR) inhibitors have been associated with increased cardiovascular disease risk due to endothelial dysfunction, hyperlipidemia and diabetes in transplant patients [1,2]. Functional or morphological change of the endothelium is the stimulus for atherogenesis, and evidence suggests that endothelial cell dysfunction is the first step in this process [3,4]. In combination with an increased risk of cardiovascular disease, a number of immunosuppressants are also associated with increased nephrotoxicity [5–7]. While nonoxidative pathways, including inflammation, are implicated in nephrotoxicity [8], there is evidence to suggest that reducing oxidative stress may reduce vascular dysfunction [9].

Given the increased risk of cardiovascular disease in those receiving immunosuppressive therapy [1], a greater understanding of the impact of immunosuppressive drugs on vascular function is warranted. To date, differences in study protocols have made it difficult to compare the effects of these treatments. The effect of immunosuppressants on vascular function, inflammation and oxidative stress has not been determined within the same model, and it remains unknown as to which drug (if any) poses the least risk for the development of vascular dysfunction.

The aim of the present investigation was to determine the effect of administration of calcineurin inhibitors (cyclosporine A and tacrolimus) and mTOR inhibitors (sirolimus and everolimus) on Wistar rat aortic smooth muscle and endothelial function. Also, markers of glomerular filtration, lipid peroxidation and antioxidant defences and inflammatory cytokines were measured.

Materials and methods

Animals and experimental overview

Forty-seven 8-week-old male Wistar rats were randomly allocated to one of six groups to undergo 10 days of therapy: [1] 10 mg/kg cyclosporine A ($n = 10$) (low dose), [10] 25 mg/kg cyclosporine A ($n = 9$) (high dose), [3] 1.0 mg/kg tacrolimus ($n = 9$), [4] 2.5 mg/kg everolimus ($n = 10$), [5] 0.4 mg/kg sirolimus ($n = 10$) or [6] placebo ($n = 9$). Cyclosporine, tacrolimus and sirolimus were administered via intraperitoneal injection, whereas everolimus was administered via gavage. We had two placebo groups during the investigation to account for potentially different effects of the drug vehicles used (intraperitoneal injection delivery of olive oil and ethanol and gavage delivery of microemulsion used for everolimus). As we did not find any differences between our two control groups, we have presented the control group data for the cyclosporine vehicle only (olive oil and ethanol). Drug doses chosen represented those associated with therapeutic doses in animals [9,11–13]. Maximum immunosuppressive effect of sirolimus in a rat model has previously been reported with an intraperitoneal dose of 0.4 mg/kg per day when compared with 0.2 and 0.8 mg/kg per day [14] and 1 week of sirolimus treatment at this dose was associated with nephrotoxicity reflective of calcineurin inhibitor therapy. Importantly, dosing at this level has also been associated with elevated tissue concentrations of the drug [15], reflects trough levels reported in rats (0.8–7.4 ng/ml) and is similar to whole blood trough concentrations in pigs (0.5–6.4 ng/ml) during oral sirolimus treatment at doses between 0.1 and 2.0 mg/kg per day [16]. For cyclosporine, we recognize that the lower dose is associated with nephrotoxicity, and for this reason, we did not solely use a 25 mg/kg/day dose which is more damaging. Our group has previously shown that 25 mg/kg of Cyclosporine A administration for 7 days resulted in a significant increase in plasma malondialdehyde, methaemoglobin and superoxide dismutase (SOD) and catalase activities [17]. A 10 mg/kg per day dose of cyclosporine A for the same duration has been shown to reduce kidney function [18] and for this reason, we included both high dose and low dose cyclosporine A groups in the current investigation. All rats were fed rat chow and water *ad libitum* and maintained on a 12-h light/dark photo-period with a room temperature of 21 ± 2 °C. Animal body weight was recorded daily, and drug doses were adjusted according to changes in body weight. Dosing of each drug was performed at approximately the same time each day. On the 10th day, animals were sacrificed. Rats were administered sodium pentobarbital (90 mg/kg) via intraperitoneal injection. After reaching a surgical plane of anaesthesia, the abdominal cavity was opened, and rats were exsanguinated via the abdominal aorta. Approximately 4 ml of blood was collected in EDTA tubes. Erythrocytes and plasma were stored at −80 °C until biochemical analysis. All experimental protocols were approved by the Animal Experimentation Ethics Committee of The University of Queensland.

Aortic vascular function

All experiments were conducted by the same technician, in the same laboratory, using the same equipment. Aortic vascular endothelial and smooth muscle function were determined *ex vivo* in organ baths according to the methods of Lexis *et al.* [9] where the isometric tension changes of aortic rings were recorded. All vasoactive drugs [noradrenaline (NA), acetylcholine (Ach) and sodium nitroprusside (SNP)] were purchased from Sigma (Sydney, NSW, Australia). The change in isometric force was measured by Grass FT03 force transducers (Grass, MA) connected to a PowerLab chart recording system using Chart 4.0 recording software (AD Instruments, Sydney, NSW, Australia).

Final drug concentrations

Whole blood cyclosporine A concentrations were determined using a cloned enzyme donor immunoassay with microgenics reagent kit (Microgenics, CA, USA) on an Abbott Architect C8000 (Abbott Laboratories, Illinois, USA). Everolimus concentration was determined using the Seradyn Innofluor Certican fluorescence polarization immunoassay run on an Abbott IMx (Abbott Laboratories, Illinois, USA). Tacrolimus and sirolimus concentrations were determined using a microparticle enzyme immunoassay (Abbott Laboratories, Illinois, USA) run on an Abbott IMx (Abbott Laboratories, Illinois, USA).

Oxidative stress/antioxidants

Oxidative stress was quantified by measuring plasma concentrations of F_2-isoprostanes (8-iso-$PGF_{2\alpha}$) and malondialdehyde. Isoprostanes were extracted and derivitized according to the methods of Taylor *et al.* [19] and Mori *et al.* [20] respectively. Samples were analysed using a Varian 320 MS/MS with a Varian 450 gas chromatograph equipped with a CP8400 auto sampler using Varian MS

Workstation – System control software version 6.9.1 (Agilent Technologies, CA, USA). Malondialdehyde was measured via HPLC (Shimadzu, Kyoto, Japan) using the method of Sim *et al.* [21].

The activities of glutathione peroxidase (GPX), SOD and catalase were determined according to the methods of Wheeler *et al.* [22], Madesh and Balasubramanian [23] and Slaughter and O'Brien [24], respectively. The assays were modified to be performed on a Cobas Mira automated spectrophotometer (Roche Diagnostics, Switzerland). All enzyme activities were normalized to haemoglobin concentration. Total antioxidant status (TAS) was determined using the method of Miller *et al.* [25] The assay was carried out on the Cobas Mira automated spectrophotometer.

Inflammation

Plasma tumour necrosis factor (TNF)-α and interleukin (IL)-1β concentrations were quantitatively determined using a rat cytokine Linco*plex* Kit (RCYTO-80K; Millipore, MA, USA). The assay was performed according to the manufacturer's instructions and analysed on a Luminex®100 (Luminex Corporation, Austin, TX, USA). The interassay coefficient of variation for TNF-α and IL-1β were 7.6% and 6.3% respectively. TNF-α and IL-1β concentrations were not determined for the high dose cyclosporine A group.

Plasma creatinine

Plasma creatinine was determined as a marker of kidney function using the Jaffe reaction method. Absorbance was measured at 520 nm on a Cobas Mira automated spectrophotometer.

Statistical analysis

Comparisons of body weight and biochemical data between drug groups were made using one-way ANOVA. If statistical significance was attained, a Bonferroni *post hoc* test was used. $P < 0.05$ was considered statistically significant. Aortic vascular function data are presented as the mean ± standard error of the mean (SEM). Comparisons between groups were made by performing nonlinear regression analysis with variable slope and least squares fit. Nonoverlapping 95% confidence intervals for either maximum contraction or relaxation were considered significantly different. Within treatment comparison was performed using two way ANOVA (group x concentration) with Bonferroni correction. Significance was set at $P < 0.05$. Data were analysed using Prism 5 for Windows (GraphPad Software, Inc., CA, USA).

Results

Drug concentration and body weight

Mean blood drug concentrations in the treated animals during sacrifice (2 h following final dose administered) were 10 mg/kg cyclosporine A = 2924 ± 303 μg/l, 25 mg/kg cyclosporine A = 5512 ± 497 μg/l, 0.4 mg/kg sirolimus = 2.03 ± 0.19 μg/l, 1.0 mg/kg tacrolimus = 47.9 ± 6.0 μg/l and 2.5 mg/kg everolimus = 14.3 ± 1.0 μg/l. Body weight increased in all groups during the experimental period

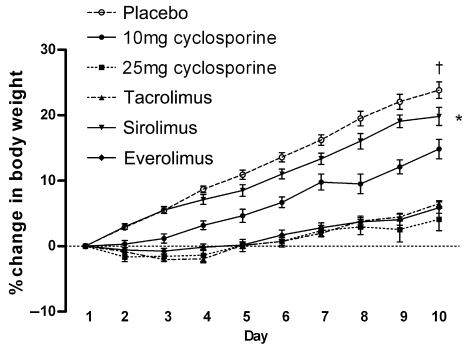

Figure 1 Change in body weight over 10 days of immunosuppressant therapy. *Significantly different from low and high dose cyclosporine A, everolimus and tacrolimus. †Significantly different from all other groups. Data are mean ± SE.

Table 1. Maximal aortic contraction or relaxation and logEC50 following 10 days of immunosuppressive treatment.

	NA		SNP		Ach	
Drug	Emax	pLog EC50	pEmin	pLog IC50	pEMin	pLog IC50
Placebo	8.494 (6.79–10.20)	8.22 (10.36–6.07)	11.37 (12.13–10.61)	7.32 (7.52–7.13)	5.49 (5.99–4.98)	6.65 (6.83–6.43)
10 mg Cyclosporine	6.74 (5.85–7.63)	6.74 (7.97–6.83)	10.89 (12.25–9.53)	6.52 (6.77–6.26)	3.36 (3.85–2.87)	7.15 (7.53–6.76)
25 mg Cyclosporine	6.22 (5.21–7.23)	7.40 (7.97–6.83)	9.76 (10.93–8.58)	7.21 (7.55–6.87)	3.26 (3.83–2.69)	6.88 (−7.29–6.48)
Tacrolimus	6.74 (5.05–10.33)	8.45 (14.06–2.84)	7.74 (8.90–6.58)	6.85 (7.20–6.49)	3.14 (3.77–2.52)	6.78 (7.26–6.31
Sirolimus	11.27 (10.17–12.38)	7.77 (8.21–7.33)	10.56 (11.76–9.36)	8.85 (8.88–7.32)	3.83 (4.79–2.87)	6.83 (7.42–6.25)
Everolimus	7.23 (6.18–8.28)	8.03 (9.34–6.72)	10.78 (12.16–9.41)	7.18 (7.54–6.83)	5.21 (6.32–4.10)	6.75 (7.25–6.26)

Ach, acetylcholine; NA, noradrenaline; SNP, sodium nitroprusside.
Data are presented as mean (95% confidence interval).

($P < 0.001$), and there was a significant difference between groups across the 10 days ($P < 0.001$) (Fig. 1).

Aortic contraction

Maximal aortic contraction in response to NA was significantly increased in the sirolimus group when compared with low and high dose cyclosporine A and everolimus (Table 1, Fig. 2a). At NA concentrations greater than 3 μM, smooth muscle contraction was increased in the sirolimus group when compared with the placebo group ($P < 0.05$) There were no significant differences in contractile responses between other drug groups and the placebo group.

Aortic endothelial-dependent relaxation

Administration of low and high dose cyclosporine A and tacrolimus resulted in reduced maximal aortic endothelial-dependent relaxation responses to ACh when compared with everolimus. Endothelial-dependent relaxation responses were similar for everolimus and placebo animals, whereas, tacrolimus, cyclosporine A (high and low dose) and sirolimus administration resulted in reduced maximal endothelial-dependent relaxation responses when compared with the placebo group (Table 1, Fig. 2b). Low and high dose cyclosporine impaired endothelial-dependent relaxation at ACh concentrations greater than 0.3 and 3 μM, respectively ($P < 0.01$), when compared with the placebo group. Tacrolimus was also associated with impaired endothelial-dependent relaxation at ACh concentrations greater than 1 μM ($P < 0.05$), compared with the everolimus and placebo groups.

Aortic endothelial-independent relaxation

Maximal endothelial-independent relaxation (SNP) was reduced following 10 days of tacrolimus administration compared with low dose cyclosporine A, everolimus and sirolimus (Table 1, Fig. 2c). Indeed, at all SNP concentrations greater than 0.1 μM, aortic relaxation was significantly impaired in the tacrolimus group. In contrast, sirolimus did not affect maximum SNP-induced relaxation, but significantly enhanced relaxation in response to 3–30 nM SNP compared with all other drug treatments ($P < 0.05$).

Antioxidant enzyme activity and oxidative stress

There was a significant main effect of treatment on erythrocyte antioxidant enzyme activity (catalase, $P < 0.001$; SOD, $P < 0.0001$; GPX, $P = 0.022$) (Fig. 3). Sirolimus was associated with significantly higher catalase activity

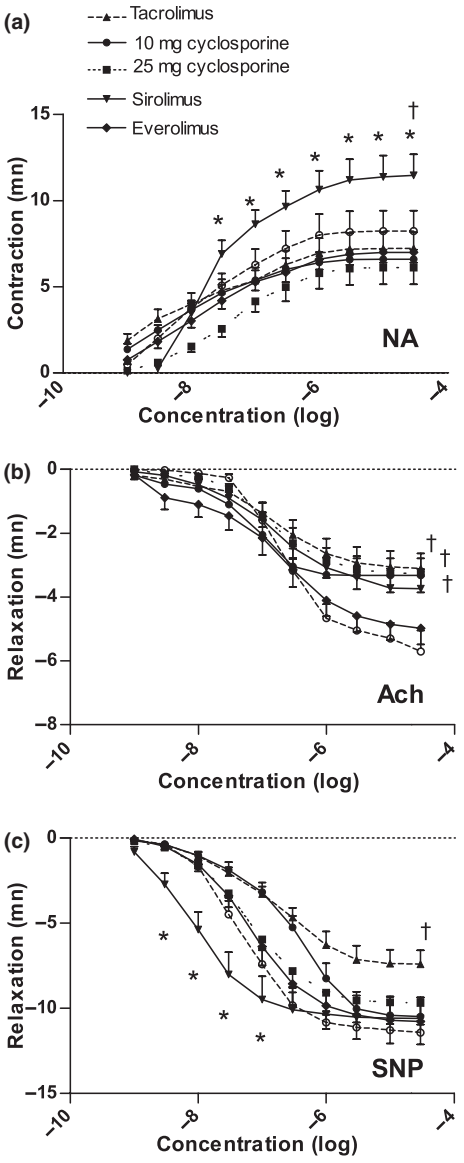

Figure 2 (a) Aortic smooth muscle contraction (NA) dose response curve. *Significantly different from all other drug groups (not placebo). †Maximal contraction significantly different from everolimus and 10 and 25 mg/kg cyclosporine. (b) Endothelial-dependent relaxation (Ach) dose response curve. †Maximal relaxation significantly different from everolimus and placebo. (c) Endothelial-independent (SNP) relaxation dose response curve. *Significantly different from all other groups. †Maximal relaxation significantly different from low dose cyclosporine A, everolimus and sirolimus. Data are mean ± SE.

compared with all other drug groups and the placebo group ($P < 0.0001$) (Fig. 3c). There were no significant differences in catalase activity for the tacrolimus, everolimus or cyclosporine A groups compared with the placebo group ($P > 0.05$). SOD activity of the sirolimus group was significantly greater than the high dose cyclosporine A group ($P < 0.01$). Low dose cyclosporine A was

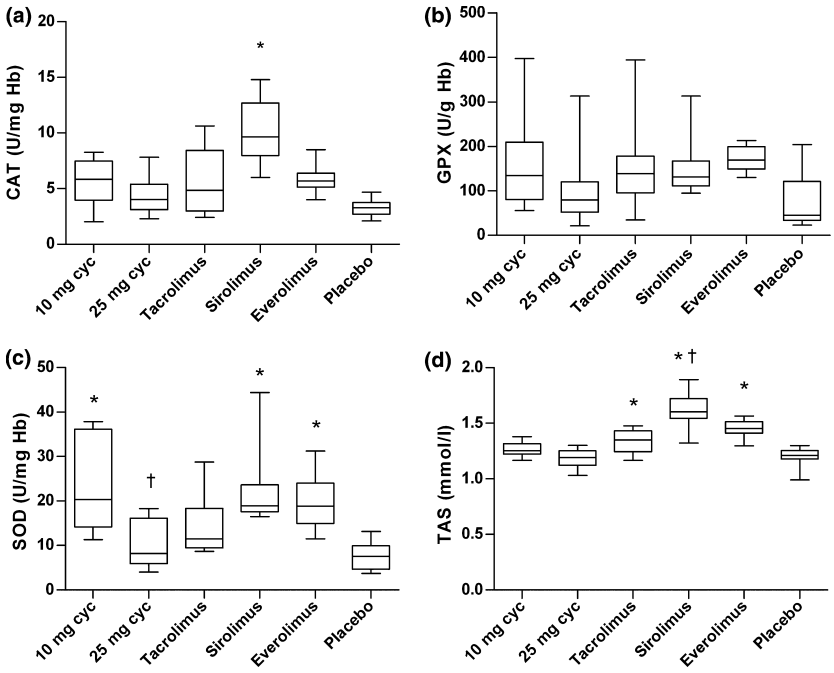

Figure 3 (a) Catalase activity. *Significantly different from all other groups ($P < 0.0001$). (b) GPX activity. (c) SOD activity. *Significantly different from placebo group. †Significantly different from sirolimus and 10 mg/kg cyclosporine groups. (d) Total antioxidant status. *Significantly different from placebo group. †Significantly different from all other drug groups. Plots represent mean ± 95% confidence interval.

associated with a significantly higher SOD activity compared with the high dose cyclosporine A group ($P < 0.01$). SOD activity was significantly greater in the low dose cyclosporine A, sirolimus and everolimus groups compared with the placebo ($P < 0.01$). While there was a significant main effect of group on GPX activity, there were no significant *post hoc* tests.

TAS was significantly different across groups ($P < 0.001$) (Fig. 3d). Sirolimus, tacrolimus and everolimus were associated with greater TAS values than the placebo group ($P < 0.01$). The mean TAS value of the sirolimus group was significantly higher than all other drug groups and the placebo group ($P < 0.01$). Low dose cyclosporine A was associated with significantly lower TAS values than tacrolimus and everolimus ($P < 0.01$), whereas the everolimus group TAS value was significantly higher than both cyclosporine A groups ($P < 0.001$).

There was a significant effect of treatment on F_2-isoprostanes ($P < 0.001$). F_2-isoprostanes values of the everolimus group were significantly greater than low ($P < 0.01$) and high dose ($P < 0.001$) cyclosporine A groups and the sirolimus group ($P < 0.01$) (Fig. 4a). Plasma concentrations of malondialdehyde were not significantly different between groups ($P > 0.05$) (Fig. 4b).

Cytokine concentrations

Sirolimus was associated with significantly lower plasma IL-1β concentration compared with everolimus ($P < 0.05$). There were no significant differences between everolimus and other drug groups or between drug groups and the placebo group (Fig. 5a).

Plasma concentrations of TNF-α were significantly lower following sirolimus compared with tacrolimus and everolimus ($P < 0.05$). Plasma TNF-α level was also significantly lower following sirolimus compared with the placebo group ($P < 0.05$) (Fig. 5b).

Creatinine

Creatinine concentration was significantly elevated following 10 days of 25 mg cyclosporine A administration compared with sirolimus ($P < 0.001$) and tacrolimus ($P < 0.01$) administration (Fig. 6). Everolimus was not significantly different from other drug groups or the placebo.

Discussion

This is the first investigation to determine the acute effects of four commonly used immunosuppressants on

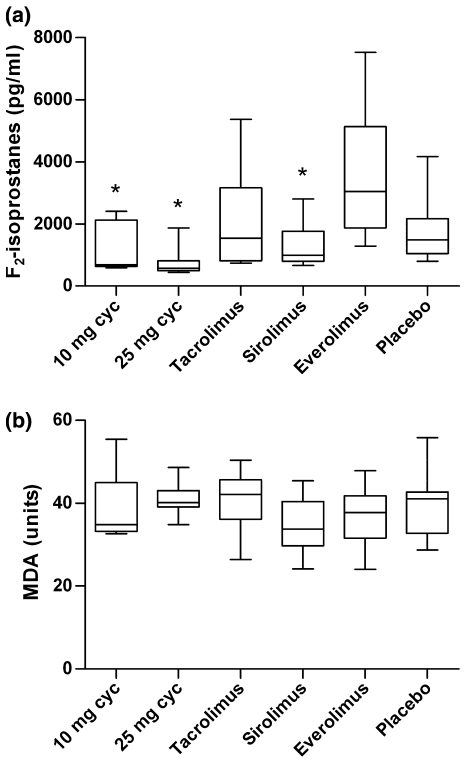

Figure 4 (a) Plasma F_2-isoprostanes. *Significantly different from everolimus group. (b) MDA. Plots represent mean ± 95% confidence interval.

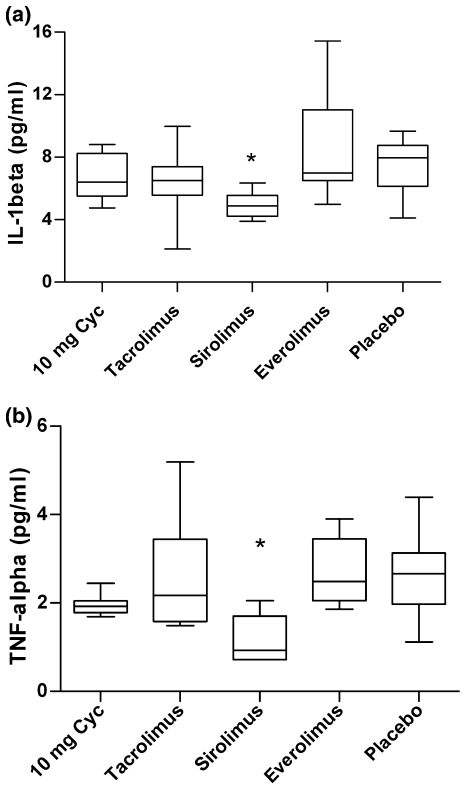

Figure 5 (a) IL-1β. *Significantly different from everolimus group. (b) TNF-α. *Significantly different from tacrolimus, everolimus and placebo groups. Plots represent mean ± 95% confidence interval.

aortic smooth muscle and endothelial function, systemic oxidative stress and inflammation in rats. Blood concentrations of each drug were at therapeutic levels (as previously reported in the literature); however, the study findings are relevant only in the context of the current experimental model and doses administered [9,11,12]. While endothelium-dependent and/or -independent relaxation was impaired following cyclosporine A, tacrolimus and sirolimus, endothelial or smooth muscle function was not compromised by everolimus. Impaired vascular relaxation is a precursor to cardiovascular pathology [26,27], and as such, comparison of drugs in post-transplant therapy requires further investigation.

Hyper-responsiveness of the aorta to NA may be attributable to altered endothelial function or the production of vasoconstrictor prostanoids as a result of oxygen-derived free radicals. The present study showed that maximal contractile responses to NA were significantly enhanced following sirolimus administration. Elevated TAS may be indicative of elevated radical production, and indeed H_2O_2 produced from the conversion of superoxide by SOD is recognized as an endothelium derived contracting factor [28]. As reactive oxygen species were not quantified in the present investigation, the precise

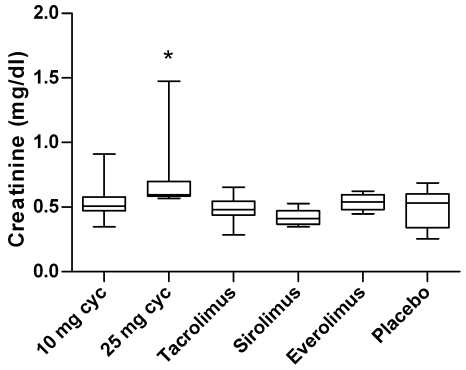

Figure 6 Plasma creatinine. *Significantly different from sirolimus and tacrolimus groups. Plots represent mean ± 95% confidence interval.

mechanism for NA hyper-responsiveness remains to be determined.

Endothelium-dependent relaxation was also impaired following administration of sirolimus. Sirolimus eluting stenting has been associated with impaired endothelium-dependent relaxation, a reduction in NO bioavailability

and increased production of reactive oxygen species [29]. The observed arterial hyper-responsiveness following sirolimus may be attributable to impaired NO signalling, and may also explain the impaired endothelium-dependent relaxation in this group.

There was no impairment of *ex vivo* smooth muscle contraction following administration of cyclosporine A, tacrolimus or everolimus. Treatment with cyclosporine A has previously been associated with arterial hypertension and elevated augmentation index [30,31], that may be mediated via an increase in protein kinase C which inhibits endothelial NO [32]. No *ex vivo* hyper-responsiveness of the aorta in low or high dose cyclosporine A-treated rats was observed in the present study, supporting the findings of Lexis *et al.* [9] who showed that 10 days of cyclosporine A does not potentiate NA-induced contraction of the rat aorta. Our findings also support those of Can *et al.* [33], who reported that 14 days of tacrolimus (1 mg/kg per day) did not influence aortic contraction *in vitro*. While our findings in relation to the effects of cyclosporine on smooth muscle contraction are similar to Lexis *et al.* [9] those in relation to endothelial-independent contraction were not. The potential differences in aortic function and antioxidant enzymes between the studies may be attributable to the different rat strains (Sprague Dawley [9] compared with Wistar in the present study). Endothelial dysfunction caused by proteinuria [34] and cellular production of oxidants [35] is strain specific, and it is therefore possible that immunosuppressant drugs exert differential effects between strains, given the strain-dependent differences in physiology. These findings highlight that strain specific vascular function requires further investigation.

Supraclinical and clinical doses of tacrolimus have been associated with endothelial dysfunction in both animal models and human transplant recipients [36,37]. Recently, Can *et al.* [33] showed that 14 days of tacrolimus therapy in Wistar rats (1 mg/kg per day) is associated with impaired relaxation responses to ACh. The present study supports these findings. Impaired endothelial-dependent relaxation of mouse aorta following *in vitro* application of tacrolimus has been linked to altered intracellular calcium release affecting eNOS [38]. Cyclosporine A has been shown to inhibit inducible NO synthase induction at the mRNA level in cultured rat endothelial cells (nonspecified strain) [39], and may be a potential mechanism for the observed impairment of endothelial-dependent relaxation following cyclosporine A. Cyclosporine A has also been shown to impair Ach-induced aortic relaxation (in a Lewis rat) due to a decrease in smooth muscle intracellular cGMP, which inhibits calcium entry to the cell [40]. While cyclosporine A, sirolimus and tacrolimus impaired aortic relaxation, everolimus was not associated with impaired vascular function. Despite sirolimus and everolimus being structurally related, everolimus is more hydrophilic with a shorter half-life and greater bioavailability compared with sirolimus [41]. This article is not the first to report differences in physiological variables between the drugs. There is evidence to suggest that everolimus is associated with improved blood lipid profile when compared with sirolimus [42], that patients on everolimus eluting stents have reportedly lower rates of myocardial infarction and target vessel revascularization when compared with those on sirolimus eluting stents [43], and that when sirolimus and everolimus are paired with cyclosporine, they exert differential effects on mitochondrial metabolism in the brain [44]. Although structurally related, everolimus and sirolimus have been shown to influence physiological variables differently.

Everolimus was, however, associated with were significantly elevated systemic F_2-isoprostanes compared with cyclosporine A and sirolimus. Concentrations of malondialdehyde were unaffected by calcineurin and mTOR immunotherapy, highlighting the sensitivity of F_2-isoprostanes as a marker of lipid peroxidation when compared with malondialdehyde. The impact of long term elevated systemic F_2-isoprostane concentrations on vascular function is yet to be determined.

In combination with impaired endothelium-dependent relaxation, tacrolimus impaired endothelium-independent relaxation compared with low dose cyclosporine A, everolimus and sirolimus. SNP induces vascular relaxation as a donor of NO, whose release from SNP is enzyme dependent. Recently, Bonaventura *et al.* [45] showed that SNP-induced relaxation is potentiated in endothelial intact aorta segments from Wistar rats when compared with denuded segments. This suggests that vessels with unimpaired relaxation in response to ACh (functioning endothelium) may have a potentiated response to SNP. Precontracted denuded human radial arteries have been shown to relax in response to increasing sirolimus concentrations *in vitro*, mediated by smooth muscle ATP-K^+ channels [46]. While sirolimus was not directly added to organ baths in the present study, endothelium-independent relaxation was greatest in the sirolimus group when compared to other drug groups. The lower levels of inflammatory cytokines observed following sirolimus may also be responsible for enhanced endothelium-independent relaxation. A reduction in TNF-α and IL-6 mRNA has previously been shown in human vascular smooth muscle cells following exposure to sirolimus [47] and this reduced expression would enhance the effect of NO on vascular relaxation.

Enhanced relaxation in response to SNP in the sirolimus group may also be attributable to higher levels of SOD. SOD has previously been shown to restore SNP-induced

relaxation in the diabetic Wistar rat [48]. In the present study, SOD activity was increased following everolimus, sirolimus and low dose cyclosporine A. Tacrolimus was not associated with an increase in SOD activity, and this group experienced impaired endothelium-dependent relaxation.

Chronic calcineurin use is associated with morphological changes of the kidney and a decline in kidney function [49]. Serum creatinine levels have previously been reported to be significantly elevated following 6 days of cyclosporine administration at 25 mg/kg per day compared with control rats, whereas sirolimus administered at 1 mg/kg per day did not affect creatinine concentration [50]. In this study, plasma creatinine concentration was significantly elevated following high dose cyclosporine compared with sirolimus and tacrolimus. High dose cyclosporine A was the only immunotherapy not associated with an increase in SOD or TAS. Alterations in kidney function may be independent of oxidative stress; however, SOD activity of the cyclosporine group was significantly lower than the sirolimus group. SOD activity may be protective against kidney dysfunction as elevated SOD activity has been shown to be protective against acetaminophen-induced nephrotoxicity [51].

Vascular dysfunction was evident following acute administration of some immunosuppressants that has implications for the development of pathologies such as cardiovascular disease. While this investigation investigated the effect of immunosuppressants on vascular function and oxidative stress in isolation, it is important to consider the influence of organ transplant on these measures and dosing effects, and also to consider application of these results to humans. It is necessary to state that the clinical efficacy of these drugs should not be ignored, and therefore a combination of these immunotherapies and different doses, which may be superior in treatment efficacy, should also be investigated, and as such clinical outcome studies comparing these agents and varying doses are required. The present data and data from trials such as the ASCERTAIN trial comparing combined everolimus and calcineurin therapy [52] may prove valuable in informing post-transplant immunotherapy.

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